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TITLE: DEVELOPMENT OF A CONTROLLED RELEASE DELIVERY SYSTEM FOR IMMUNOGENS

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SMALL BUSINESS INNOVATION RESEARCH PROGRAM
PHASE 1 – FY 1989
PROJECT SUMMARY

Topic No. A89-081 Military Department/Agency U.S. Army

Name and Address of Proposing Small Business Firm

DynaGen, Inc.
99 Erie Street
Cambridge, MA 02139

Name and Title of Principal Investigator

Judith P. Kitchell, Ph.D., Manager, Biomaterials Programs

Proposal Title

Development of a Controlled Release Delivery System for Immunogens

Technical Abstract (Limit your abstract to 200 words with no classified or proprietary information/data.)

The development program addresses the problem of effectively protecting military personnel from bacterial, viral, or toxic incapacitation. At the present, logistical problems in carrying out a protective program remain formidable because vaccination programs involve: multiple injections; administration by trained medical personnel; and careful medical record maintenance. An approach to reducing the logistical problems is to modify immunization formulations so that single doses confer complete immunity.

The overall program goal is to demonstrate that immunity comparable to that obtained with an initial vaccination plus one booster vaccination can be obtained by simultaneous injection of normal and "sleeper" vaccine formulations. In Phase I of the program, prototype formulations demonstrated the feasibility of three important facets of the "sleeper" concept. One formulation (which did not contain protein) showed that, when subjected to a moist environment, it could remain almost unchanged for about 6 weeks, at which point it rapidly swelled and released many small particles. Another prototype, containing protein, showed delayed protein release properties; and yet a third formulation was used to demonstrate retention of antigenic conformation after processing and exposure to moisture for two weeks.

Anticipated Benefits/Potential Commercial Applications of the Research or Development

The proposed controlled release delivery system will provide a higher degree of immunity with only a single administration of vaccine. This will eliminate the need for booster immunizations which presents a major logistics problem in military use. Additionally, in the more routine, non-military indications, multiple dose vaccination programs are difficult to implement in parts of the world where the need is often the greatest. Economic, cultural, and/or geographic factors are major obstacles in obtaining repeated access to individuals at risk.

List a maximum of 8 Key Words that describe the Project.

controlled release, immunization, copolymers, poly(lactic/glycolic) acid (PLGA), vaccine, matrix, microparticles, inoculation

DEVELOPMENT OF A CONTROLLED RELEASE DELIVERY SYSTEM FOR IMMUNOGENS

FINAL REPORT FOR PHASE I

DynaGen, Inc. Report No. 2480
Contract DAMD-17-89-C-9141

Covering the period:

August 15, 1989 to February 15, 1990

The views, opinions and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

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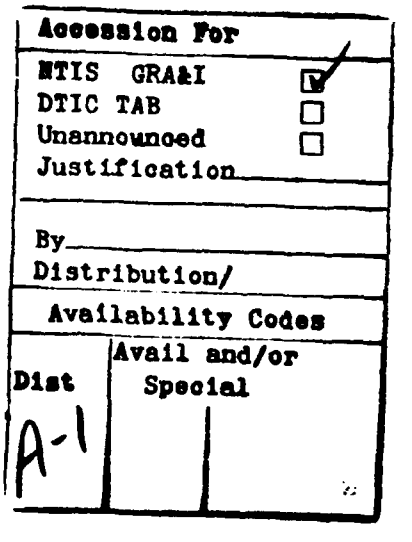

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ABBREVIATIONS

AHH	Aluminum Hydroxide Hydrate
BSA	Bovine Serum Albumin
DNP-BSA	Dinitrophenol Modified Bovine Serum Albumin
HAV	Hepatitis A Virus
PBS	Phosphate Buffered Saline
PLGA	Poly(lactide/glycolide)

SUMMARY

The development program addresses the problem of effectively protecting military personnel from bacterial, viral or toxic incapacitation. The threatening source of exposure may be natural or the result of enemy action. Often the potential threat may be anticipated and personnel may be offered protection in the form of vaccination regimens. At the present, even when the danger is identified and the prophylaxis is readily available, logistical problems in carrying out a protective program remain formidable. Immunization is presently conferred through a vaccination program which involves: multiple injections; administration by trained medical personnel; and careful medical record maintenance.

An approach to reducing the logistical problems is to modify immunization formulations for each vaccine so that a single dose confers complete immunity. As it is known that the human immune system frequently requires multiple experiences of exposure to immunogens in order to build lasting immunity, the formulation must reproduce this effect by presenting not one exposure, as is made with present dose forms, but multiple exposure events. The ideal vaccine formulation will include simultaneous administration of the presently used liquid suspension, which will immediately present the recipient with the first exposure to antigen, along with one or more "sleeper" components, which will lie dormant for several weeks before presenting subsequent exposures. The individual will require only one encounter with medical personnel to receive full immunization, thus reducing time expended by medical and record maintenance personnel, and eliminating the risk of failure to receive the "booster" dose.

In the present program, a "sleeper" vaccine component is under development. The overall program goal is to demonstrate that immunity comparable to that obtained with an initial vaccination plus one booster vaccination can be obtained by simultaneous injection of normal and "sleeper" vaccine formulations. The "sleeper" vaccine was partially developed in Phase I of the program. Prototype formulations demonstrated the feasibility of three important facets of the concept. One formulation (which did not contain protein) showed that, when subjected to a moist environment, it could remain almost unchanged for about 6 weeks, at which point it rapidly swelled and released many small particles. Another prototype, containing protein, showed delayed protein release properties; and yet a third formulation was used to demonstrate the retention of antigenic conformation after processing and exposure to moisture for two weeks. We believe that with our formulation technology it will be feasible to control the length of the dormant "sleeper" period from four to eight or more weeks.

Section 1

INTRODUCTION

1.1 The Problem Addressed

The development program addresses the problem of effectively protecting military personnel from microbial, viral or toxic incapacitation. The threatening source of exposure may be natural or the result of enemy action. Often the potential threat may be anticipated and personnel may be offered protection in the form of vaccination regimens. At the present, even when the danger is identified and the prophylaxis is readily available, logistical problems in carrying out a protective program remain formidable. A protective immunization is not like a garment which can be donned quickly in a time of need. Immunization is presently conferred through a vaccination program which involves:

- multiple injections;
- administration by trained medical personnel; and
- careful medical record maintenance.

When each individual receives the initial vaccination, which should be well in advance of exposure, he or she will be treated by a trained medic and the type and date of injection will be recorded. Follow-up will be required to see that each individual meets again with a medical person to receive the necessary "booster" vaccination; more than one subsequent contact may be necessary. It must be understood that without the "booster" protection is incomplete, and persons who have been not been fully treated must not be placed in a "protected" category. In summary, major difficulties in conferring protection for large populations lie in planning and carrying out multiple medical appointments.

1.2 Proposed Solution

The solution to the problem is to modify immunization formulations for each vaccine so that a single dose confers complete immunity. As it is known that the human immune system frequently requires multiple experiences of exposure to immunogens in order to build lasting immunity, the formulation must reproduce this effect by presenting not one exposure, as is made with present dose forms, but multiple exposure events.

The ideal vaccine formulation will include simultaneous administration of the presently used liquid suspension, which will immediately present the recipient with the first exposure to antigen, along with one or more "sleeper" components, which will lie dormant for several weeks before presenting subsequent exposures. The individual will require only

one encounter with medical personnel to receive full immunization, thus reducing time expended by medical and record maintenance personnel, and eliminating the risk of failure to receive the "booster" dose.

In the present program, the "sleeper" vaccine component is to be a powder containing antigen bound in a polymer. It is important to note that the polymer is approved by the U.S. FDA for clinical uses and that it is biocompatible and fully degradable.

1.3 Summary of Phase I Results

The overall program goal is to demonstrate that immunity comparable to that obtained with an initial vaccination plus one booster vaccination can be obtained by simultaneous injection of normal and "sleeper" vaccine formulations. The "sleeper" vaccine, consisting of polymeric binder, antigen, and, in some cases, adjuvant, was partially developed in Phase I of the program. Prototype formulations demonstrated the feasibility of three important facets of the concept. The three facets are summarized below.

One important and unique formulation method demonstrated in this program is the incorporation of an adjuvant, aluminum hydroxide hydrate [AHH] in a polymer matrix. AHH is used presently as an adjuvant in vaccines because it is a tissue irritant. The AHH is presently used as liquid suspension, which is prepared from dried aluminum hydroxide hydrate gel (50 to 57.5% as Al_2O_3), a fine powder which is hydrated very slowly. In DynaGen prototypes, the AHH is incorporated as a dry powder in a degradable hydrophobic polymer matrix (which further delays hydration). The slowly swelling AHH eventually causes bursting of the matrix, thus functioning as a timer for the period of dormancy. One formulation (which did not contain protein) showed that, when subjected to a moist environment, it could remain almost unchanged for ~ 6 weeks, at which point it rapidly swelled and released many small particles.

The "sleeper" formulations have been prepared with and without the adjuvant. When no AHH is employed, the antigenic material is mixed directly with the polymer. A pulsed release of protein was obtained by this method. When the adjuvant is added, the antigenic portion of the vaccine may be incorporated into the polymeric matrix either by prior adsorption onto the aluminum hydroxide, a process utilized in the current vaccine formulation, or by inclusion as a third separate component of the matrix. A prototype containing a model protein, bovine serum albumin [BSA] and AHH showed a delayed release profile.

A third type of formulation was used to demonstrate the retention of antigenic conformation in BSA modified with dinitrophenol [DNP-BSA] during processing and exposure to moisture for two weeks. A preparation containing 4% DNP-BSA in PLGA was prepared. Collection of protein released into buffer was made over two 24 hour

periods, the initial 24 hours and also a 24 hour period following 2 weeks of storage in buffer. In both cases the samples were kept at 37°C. The results indicated that some activity was retained after 2 weeks, however it is not clear whether the reduction observed was from protein denaturation or from hydrolysis of the DNP-BSA bonds. Substantial yellow color, indicative of free DNP, was observed in the 2 week leaching buffer.

We believe that using the polymeric binder technology it will be feasible to control the length of the dormant "sleeper" period from four to eight or more weeks. In Phase II, the program will continue with the preparation of similar formulations containing immunogens. The objective in Phase II will be to incorporate the target immunogen, Hepatitis A Virus [HAV], in a biodegradable, biocompatible polymeric matrix and to demonstrate that the preparation is effective in vivo as a "sleeper" vaccine component.

Section 2

EXPERIMENTAL APPROACH

2.1 Background

2.1.1 Vaccine Formulations

To understand the program rationale, a review of some facets of vaccine formulation may be helpful. In a classical immunization regimen, a single dose of vaccine (antigen) is delivered in one injection and a short-term immunity is produced. The treatment is repeated one or more times and the subsequent exposures to antigen produce a secondary immune response which gives rise to a long-lasting immunity. In each dose, the immune response may be enhanced by the use of an "adjuvant" which magnifies the local response to the presence of foreign matter (the vaccine). One example of an adjuvant is aluminum hydroxide gel; another approach is the use of antigenic peptides.

The length of time that antigen is present as a result of one injection can be extended by utilizing an oil based medium such as Freunds' Complete Adjuvant, which degrades and releases antigen slowly [1]; however, because of degradation products, this medium is not approved for use in humans. Safer adjuvants and release-prolonging media with comparable effectiveness have been sought for many years[2].

Langer and Folkman demonstrated the first biocompatible polymeric delivery system capable of delivering proteins for prolonged periods[3]. In more recent studies, they showed that the immune response induced by sustained antigen delivery from polymers was comparable or superior to that induced with two doses in Freund's Adjuvant[4]. The polymer, poly(ethylene/vinyl)acetate, provided a sustained immune response for 25 weeks. The lack of biodegradability of this polymer limited the viability of the formulation for human usage. More recently, Langer has worked with a polymer which degrades to tyrosine derivatives as a vaccine delivery system[5]. This polymer is apparently able to

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[1] Freund, J., Am. J. Clin. Pathol. 21:645 (1951).

[2] Hilleman, O.R., Am. J. Med. 38:751 (1965).

[3] Langer, R. and Folkman, J., Nature 263:797 (1976).

[4] Langer, R. and Preis, I., J. Immunol. Methods 28:193 (1979).

[5] Kohn, J., Niemi, S.M., Albert, E.L., Murphy, J.C., Langer, R., and Fox, J.G., J.

release antigenic material for long periods and in addition the degradation products act as adjuvant.

At DynaGen, a well known biocompatible and biodegradable polymer, PLGA was utilized in a vaccine formulation including killed whole cells. As shown in Figure 2.1, this formulation released protein slowly over a period of weeks in vitro. At three weeks, the immune response induced by the slow release formulations was compared with that obtained with an equivalent dose of the standard formulation with aluminum hydroxide adjuvant. Partial efficacy was achieved even though full release, as seen in vitro was not yet achieved. These results are shown in Figure 2.2.

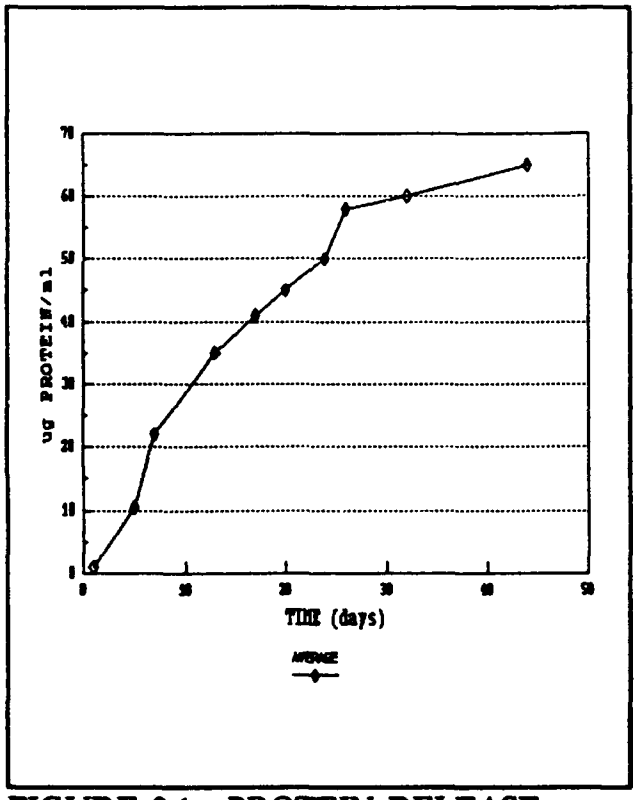

FIGURE 2.1 - PROTEIN RELEASE FROM A CELL/PLGA MATRIX

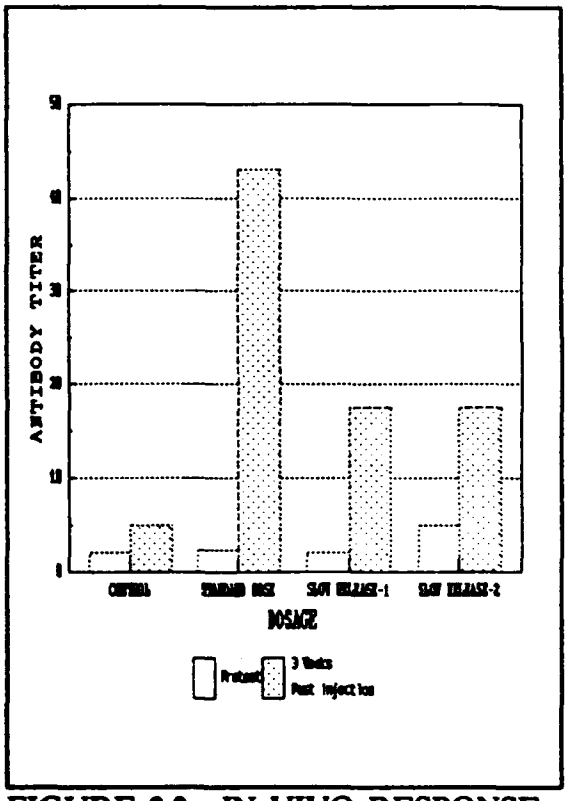

FIGURE 2.2 - IN VIVO RESPONSE TO CELL\PLGA FORMULATION

Immunol. Methods 95:31-38 (1986).

Another approach to inducing lasting immunity with one administration of vaccine is to simulate the situation of multiple exposures to the antigen as seen in the efficacy of multiple dose booster regimens. DynaGen has chosen to focus on single dose vaccines which carry "sleeper" components which deliver delayed release of antigens. The phenomenon of delayed release of compounds from formulations has been previously achieved with some small, hydrophobic compounds incorporated in polymeric matrices. The release of drug compound was very low until the polymer degraded to a certain extent, then the bulk of the drug was released in a burst. We had also observed that high molecular weight compounds such as proteins incorporated in polymer matrices would swell, causing bursting of the matrix. These observations led to the work in Phase I of this program for a single dose dual pulse vaccine.

2.1.2 Controlled Release from Degradable PLGA Polymers

The program rationale is based on extensive technology in the field known as "controlled release". It will be helpful to the reader to review the portion of this field which directly relates to this program. Biodegradable polymeric drug delivery systems have been the subject of applied research and development for approximately two decades[6,7,8,9,10&11]. Polylactic acid, polyglycolic acid, and the poly(lactic/glycolic) acid copolymers (PLGA) are known to undergo slow hydrolysis when implanted in tissue[12] and the end products of their hydrolysis (lactic and glycolic acids) are normal metabolites. For these reasons and because they exhibit moderate strength in tension, compression, and bending, they have been used for many years to manufacture absorbable sutures[13,14&15].

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[6]Jackanicz, T.M., Nash, H.A., Wise, D.L., and Gregory, J.B., Contraception 8:227 (1973).

[7]Anderson, L.C., Wise, D.L., and Howes, J.F., Contraception 13(3):375 (1976).

[8]Miller, R.A., Brady, J.M., and Cutright, D.E., J. Biomed. Mater. Res. 11:711 (1977).

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[10]Wise, D.L., Biopolymeric Controlled Release Systems (Volumes I and II), CRC Press, Inc. (1984).

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[12]Cutright, D.E., Beasley, J.D., III, and Perez, B., Oral Surg. 32:165-173 (1971).

[13]Kurkarni, R.K., Pani, K.C., Neuman, C., and Leonard, F., Arch. Surgery 93:839-843 (1966).

These same features make PLGA copolymers desirable for use in other medical, veterinary, and agricultural applications.

A substantial body of technology has been developed which takes advantage of the characteristics of polylactic and polyglycolic acids. PLGA is miscible with a wide variety of biologically active compounds. Solid formulations of polymer with biologically active compounds may be prepared and these may be designed to respond to an aqueous environment by slowly releasing the compound and degrading the polymer (by hydrolysis) to the monomers, lactic acid, and glycolic acid. The polymer breaks down chemically not only at the surface but also in the interior (homogeneous erosion). Since polylactic acid and polyglycolic acid are degraded to lactic and glycolic acids and since the end products of their hydrolysis are normal metabolites, their use as carriers of vaccines seems logical.

A significant benefit of the PLGA copolymer carrier system is evident in the ability to regulate the rate of release of active component from the PLGA matrix. The rate of release is strongly dependent on the proportion of polylactic acid and polyglycolic acid present in the matrix and the method of formation. The mechanism of release from PLGA compressed matrices is a combination of diffusion and erosion[16]. Thus, as drug (antigen) particles which are solvated diffuse from the matrix, exposed polymer hydrolyses and releases monomers. A new drug (antigen)/matrix surface is exposed and the process of diffusion and erosion continues. The degradation rates strongly depend on the mole ratio of the monomers[3] and the rate of release of a particular drug from a specific PLGA matrix can be controlled by manipulating the percentage drug content in the drug(antigen)/polymer composite[16].

Currently, several PLGA controlled release systems have been submitted as an Investigative New Drug (IND) and at least one system has been submitted as a New Drug Application (NDS). Several PLGA controlled release systems are now in Phase II testing involving several hundred human subjects, at least two products have been approved. Additionally, a PLGA controlled release formulation containing lupron, a luteinizing hormone releasing hormone [LHRH] agonist manufactured by Takeda-Abbott Pharmaceuticals, Inc., has recently received FDA NDA approval for use in the treatment of prostate cancer.

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[14]Benagiano, G., Ermini, M., Chang, C.C., Sundaram, K., and Kincl, F.A., Acta Endocrinol. 29:63 (1970).

[15]Benagiano, G., Ermini, M., Carenza, L., and Rolfini, G., Acta Endocrinol. 73:335 (1973).

[16]Kitchell, J.P. and Wise, D.L., Methods in Enzymology 112:436 (1985).

2.2 Rationale and Experimental Approach

As was described above, the utilization of PLGA in long-lasting dose formulations is well known. The choice of which PLGA to use is determined by the desired release duration. In the first booster "sleeper" formulation, the period of release will be perhaps two to six weeks, and a rapid degradation of polymer is desired. For this reason, a 50:50 mole ratio lactide/glycolide polymer was chosen. We chose a modest molecular size for the polymer, insuring that the initial degradation would be slow.

Two approaches for burst control were investigated. One tactic was essentially to continue the work described in section 2.1.1, the burying of protein in the polymer matrix. We assumed that the vaccine would consist of a soluble protein or killed organism which could be placed in a matrix, and that the material would swell and eventually burst from the matrix. In this approach, we planned to utilize a low loading of vaccine, possibly with a washing or coating to reduce the release of surface material. The incorporation would be tested using a model compound, bovine serum albumin [BSA]. We would add lyophilized protein to polymer solution, form a matrix film BSA/PLGA which would be compressed and formed into a powdered dose form. The release profile of such a powder could be evaluated by leaching powder into a buffered solution, periodic sampling, and protein concentration determination by BioRad protein assay.

The second tactic was suggested by the inclusion of aluminum hydroxide in some liquid vaccine formulations. This is the present method of delivery of the Hepatitis A virus [HAV] vaccine developed at the U.S. Army Department of Biologics. The HAV vaccine is currently formulated by adsorption on this material. We were interested in one property of the precursor dry aluminum hydroxide hydrate [AHH]: it swells very slowly in water. We thought that the AHH might swell even more slowly when incorporated in the polymer matrix, and that it would ultimately develop a hydrostatic pressure far greater than that of protein alone, thus causing a more controlled burst effect. The added advantage of this method is that some adjuvant would be needed at the time of the "sleeper" release, and while other adjuvants could be incorporated, the use of the relatively insoluble inorganic material would simplify the formulation.

To our knowledge, no combination of PLGA and AHH had been previously prepared. We were anxious to observe the swelling and bursting of AHH containing polymer matrices [AHH/PLGA] independent of protein content, and thus we prepared AHH/PLGA matrix in the form of extruded rods and ground powder. We intended to observe the physical changes in these materials with the use of the light microscope.

A separate study was designed to evaluate the processing methods for ability to retain the stability of the protein, and also to evaluate the completed matrix for ability to maintain protein stability after wetting. A model protein [dinitrophenol-bovine serum

albumin (DNP-BSA)] which has previously been utilized in immunization experiments in vivo was chosen.[17] This protein could be assayed in two ways: by the BioRad protein assay, which would measure soluble protein concentration, and by the ELISA test which would measure specifically the concentration of DNP.BSA sites. In this way the loss of DNP or the distortion of the protein near the DNP sites would decrease the concentration perceived in an ELISA test in comparison with the concentration observed in the BioRad test.

The planned accomplishments of the Phase I work were to obtain data on controlling burst time in two ways, by manipulation of protein content and method of protein incorporation, and by addition of AHH. In addition, we intended to show that the protein antigen would retain stability in the product throughout the processing and during the "sleeper" period.

[17] Little, J.R. and Eiser, H.N. in Methods in Immunol. and Immunochem. Vol 1, pp128, Nisonoff, A. Ed. Academic Press 1967.

Section 3

METHODS, RESULTS, AND DISCUSSION

3.1 Aluminum Hydroxide Suspensions

It was our intention to evaluate incorporation of AHH-HAV in the "sleeper" vaccine formulations, and there were several issues regarding the details of placing this material in a water-free polymeric matrix. An aqueous aluminum hydroxide suspension cannot be mixed with polymer solution before casting. The polymer is dissolved in methylene chloride, and we found that the aluminum hydroxide suspension and the solvent were completely immiscible. Also, adsorbed HAV is prepared as a wet suspension in buffer, and we do not want to include buffer in our final preparation unless it is essential for protein stability.

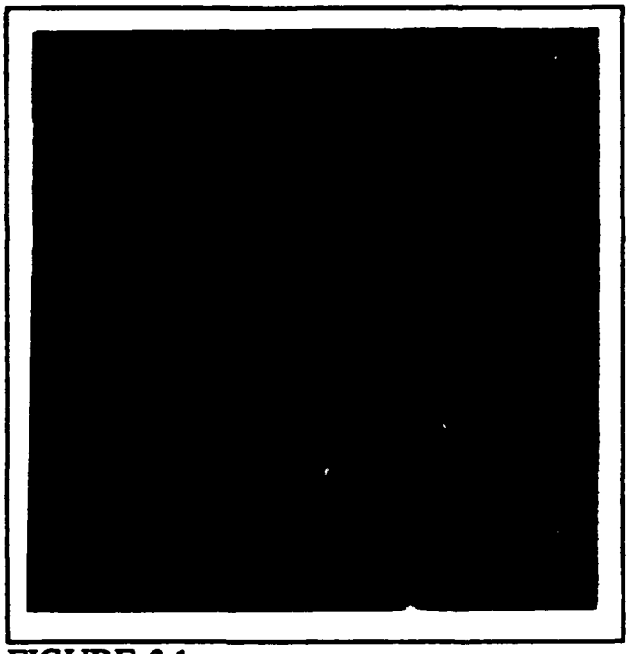


FIGURE 3.1
WASHED AHH

We wished to determine the feasibility of removing water or buffer from aluminum hydroxide suspensions. To this end, we prepared a 1 mg/ml suspension in phosphate buffered saline [PBS] (mimicking the adsorbed HAV suspension). Aluminum hydroxide hydrate was obtained from Aldrich (No. 23,918-6). We placed 1 ml of the suspension in each of 4 microcentrifuge tubes. The tubes were spun at 11,000 g for 5 minutes, then the supernatant was decanted and 1 ml of distilled water was added to each tube. The tubes were spun as before and the supernatant was again removed. This washing was repeated two more times and the aluminum hydroxide was air dried. The resultant pellet is shown in Figure 3.1.

This process is accomplished without the loss of aluminum and the dried material remains a free flowing powder. We anticipate utilizing this method of preparation (although with more rigorous drying) for AHH-HAV prior to incorporation in the matrix.

3.2 AHH/PLGA Rods and Powders

3.2.1 Preparation

Dry AHH can be suspended in polymer solution. The aluminum hydroxide hydrate/polymer [AHH/PLGA] rods and powders were prepared according to the flow chart shown in Figure 3.2. The polymer was DuPont Medisorb 50:50 D,L lactide/glycolide of inherent viscosity ~0.8 dl/g. An aliquot of 3 g polymer was added to methylene chloride and stirred. All of the polymer did not dissolve, so the sample was filtered and dried. The weight of the recovered material was 2.9718g. This recovery rate was utilized in subsequent preparations to calculate initial polymer weight. To the dry polymer, 30 ml methylene chloride was added. An aliquot of the AHH (Aldrich) [0.3309 g] was added under nitrogen. The suspension was stirred, then cast on a glass plate and spread with a Boston Bradley blade to 0.025" thickness. The casting was vacuum dried and 2.5385 g was recovered (77% yield). The casting was extruded at 55 - 65 ° C. Pliable rods were obtained and some of these were ground and sieved.

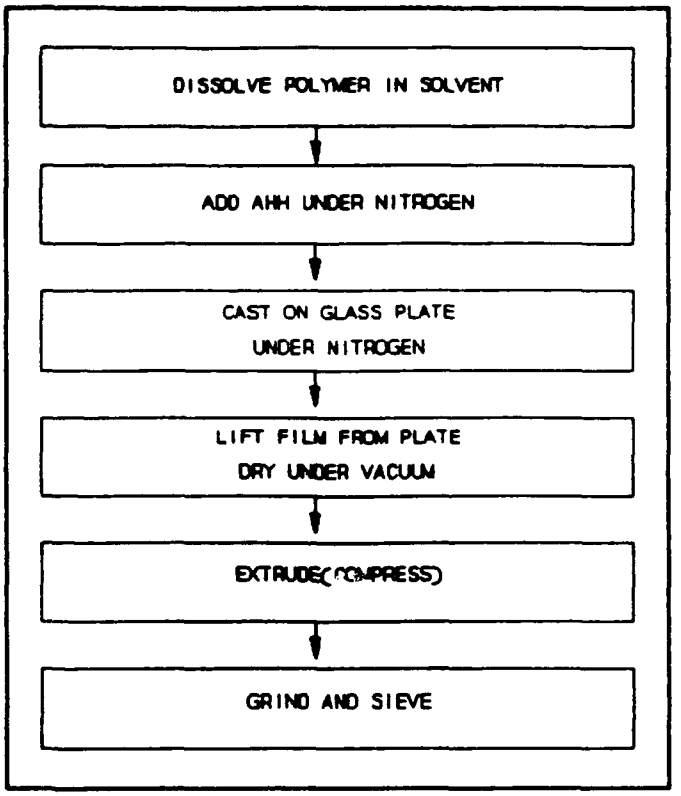


FIGURE 3.2
FLOW CHART OF AHH/POLYMER RODS AND POWDER

3.2.2 Swelling of AHH/PLGA

The appearance of the AHH/PLGA rods is shown in Figure 3.3(a). Two sections (~1 cm length) of the AHH/PLGA rods were cut and measured. The initial volumes of the rods were calculated. The rods were then placed in separate vials with PBS and maintained at 37° C. At weekly intervals the rods were removed from the vials for measurement and photo documentation.

After a rapid shrinkage, expansion in diameter and shortening in length, the rods slowly swelled. At 5 weeks, one rod broke in two. At 6 weeks, "pimples" appeared on the rod surfaces [see the upper edge of the rod in Figure 3.3(b)] and within a day these had become large bumps on the rods [see the lower edge of the rod in Figure 3.3(b)].

After another week, particles broke from the rods [Figure 3.3(c)]. The overall volume changes are shown in Figure 3.3(d).

FIGURE 3.3-CHANGES IN AHH\PLGA RODS OVER TIME

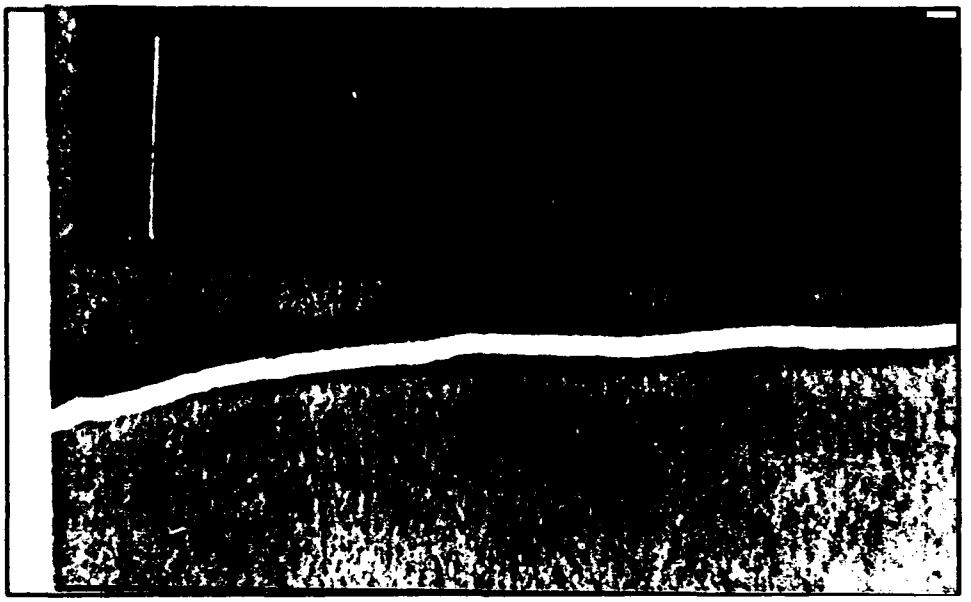


FIGURE 3.3 (a)
RODS AS EXTRUDED

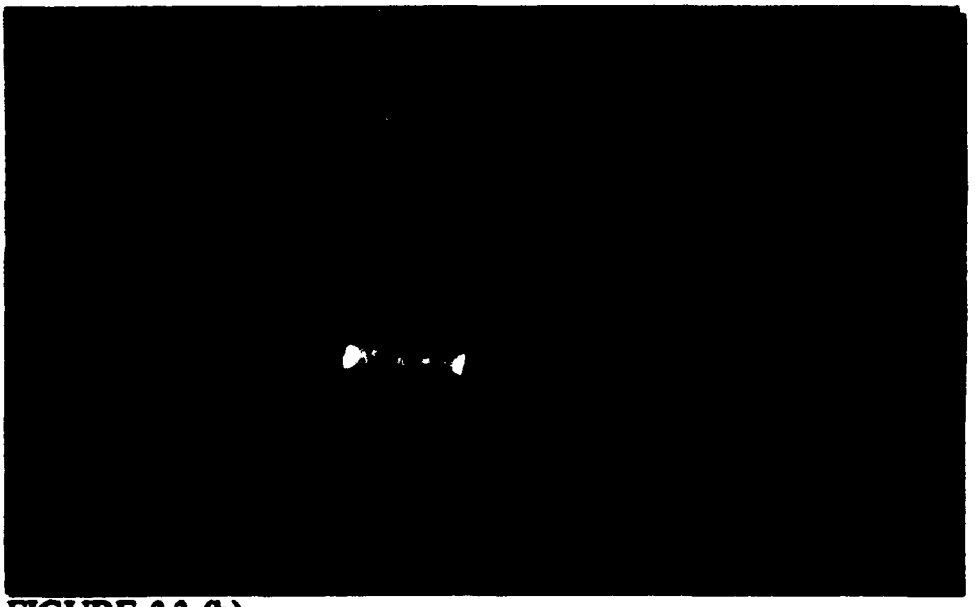


FIGURE 3.3 (b)
AHH\PLGA RODS
WITH "PIMPLES" AND BUMPS

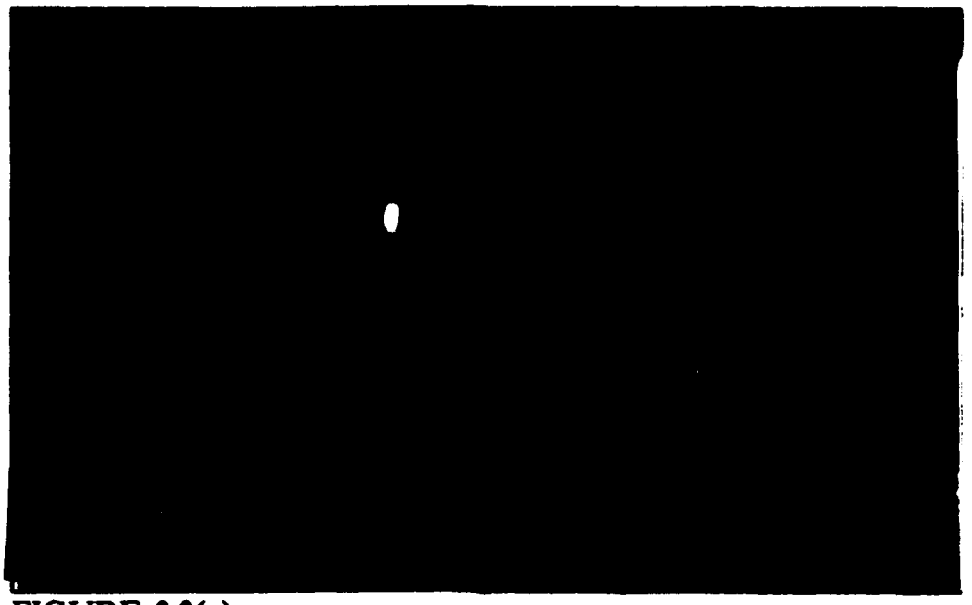


FIGURE 3.3(c)
AHH\PLGA RODS WITH PARTICLES

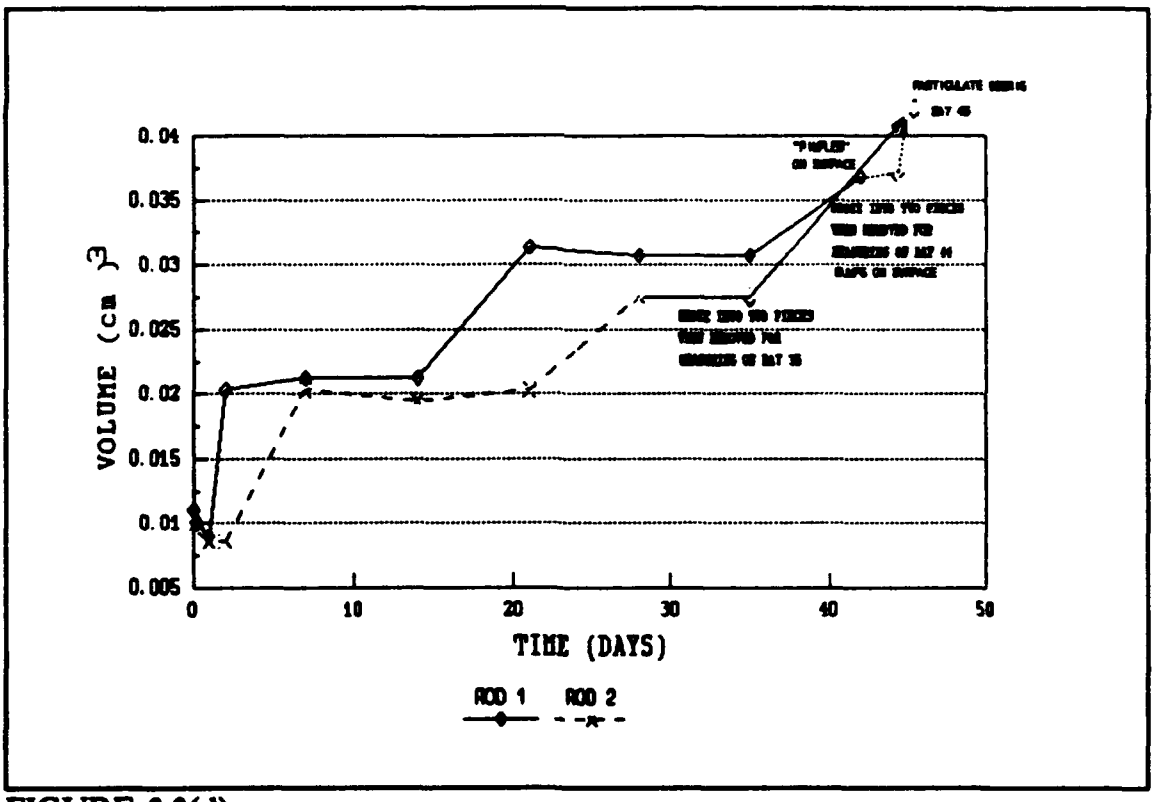


FIGURE 3.3(d)
VOLUME CHANGES IN RODS

We were curious to know if the same phenomenon would occur in the material observable on a smaller scale. Thus three particles of ground rods were placed in PBS and similarly observed and measured. The particles are shown in a watch glass in Figure 3.4 and results of size changes can be seen in Fig. 3.5. For the volume calculations, we assumed that the particles were cylindrical, as this was the closest geometry to their appearance. The particles changed most extensively at ~ 5 weeks, consistent with the results of the rod swelling study, although it should be noted that the particles accidentally dried out several times during the experiment, and the results thus may indicate a longer dormant period than is accurate.

FIGURE 3.4
AHH/PLGA PARTICLES

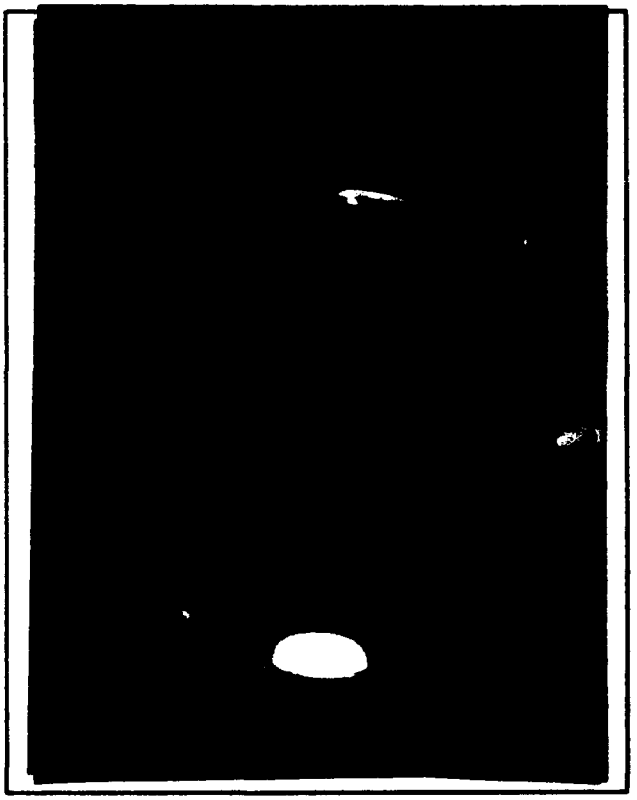
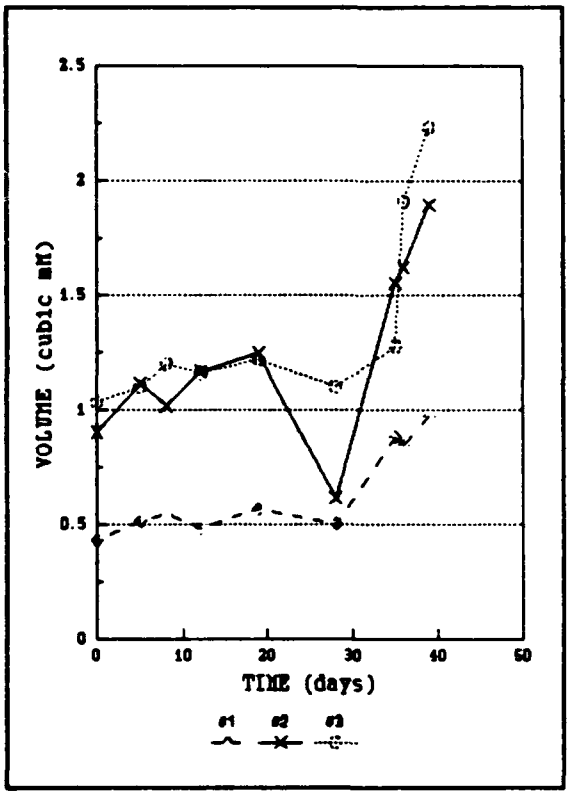


FIGURE 3.5 -
PARTICLE SIZE CHANGES



3.3 Protein Release

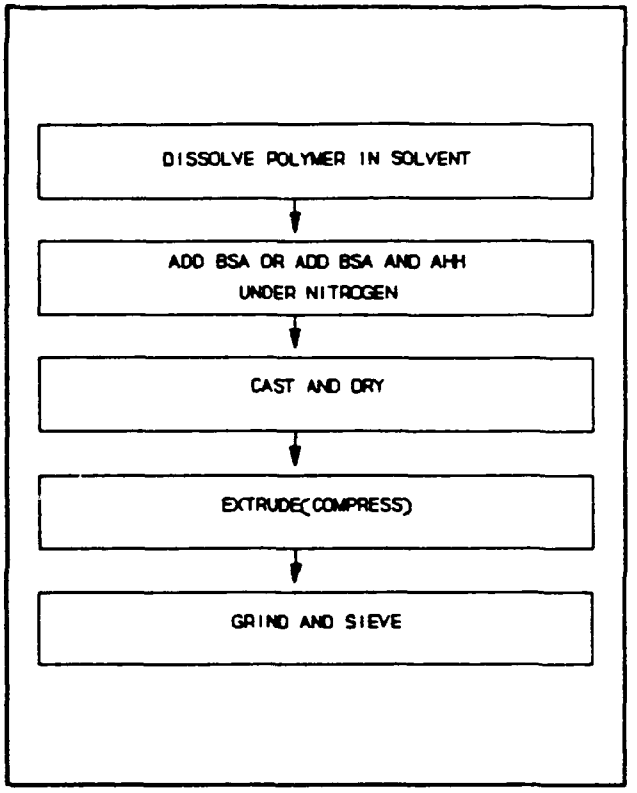
3.3.1 Matrix Preparation

All preparations followed the general outline shown in Figure 3.6.

*7.5%BSA\92.5%PLGA

Polymer, 50:50 D,L-lactide/glycolide, was obtained from DuPont (Medisorb). An aliquot of 2.6989 g was placed in a jar with 30 ml of methylene chloride. This solution was stirred overnight and then pressure filtered through a 10u polyethylene filter. We assumed an actual weight of 1.8475. A 0.1498 mg aliquot of bovine serum albumin (BSA) from Sigma (A-0281) was added and the mixture was stirred for 15 min. The protein particle size seemed large. The slurry was cast on a glass plate, spread to a thickness of ~0.025 in. The plate was then placed under nitrogen. After two hours the film was removed from the glass and dried under vacuum. The dried weight was 2.3587. Because the particles were so large, the casting was redissolved and recast. After drying the weight of recovered materials was 2.1330 g. The casting was extruded at 60-70 ° C. Portions of the rods were ground and sieved to collect particles of 106 - 250u.

FIGURE 3.6 - FLOW CHART FOR PREPARATION OF BSA CONTAINING FORMULATIONS



*4%BSA/96%PLGA

We used 2.8868 g polymer in 30 ml methylene chloride. Glass beads, to reduce particle size, and 0.1200 g BSA were added. The mixture was rolled overnight and then cast on a glass plate. The casting was dried under vacuum, then extruded at about 55 - 67 ° C. The resultant rods were ground and sieved, particles in the range 106 -250 u were collected.

*4%BSA/10%AHH/86%PLGA

An aliquot weighing 3.0005 g polymer in 30 ml of methylene-chloride was pressure filtered after a long period of dissolution (assumed final weight 2.9585 g). Aliquots of BSA (0.1374 g) and AHH (0.3455 g) were added, along with glass beads. The mixture was ball milled overnight. The next day, the suspension was cast under nitrogen (the glass beads were retained). After a few hours under nitrogen, the casting was lifted and dried further under vacuum. An amount weighing 2.5649 g was retrieved. After extrusion at 55 - 66°C and grinding and sieving, the following fractions were obtained: >250 u - 0.3898 g; 250 - 106 u - 0.4917 g; <106 u - 0.0376 g. The overall recovery was thus 0.9183 (31%).

3.3.2 Protein Release Measurements

FIGURE 3.7 - RELEASE OF BSA FROM 7.5%BSA\92.5%PLGA

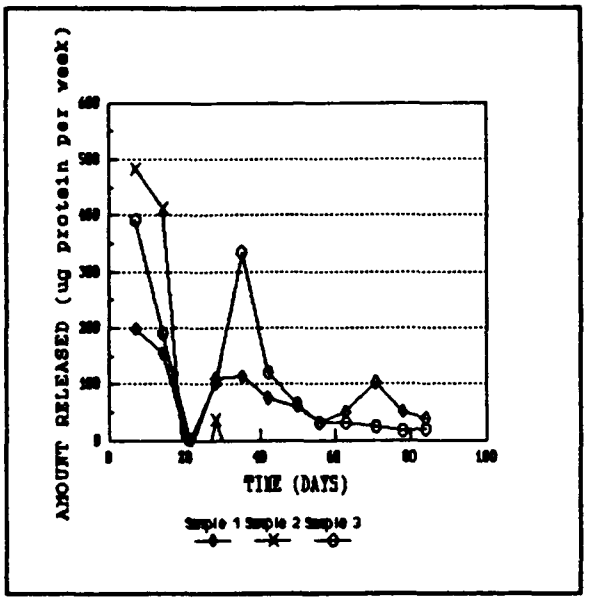
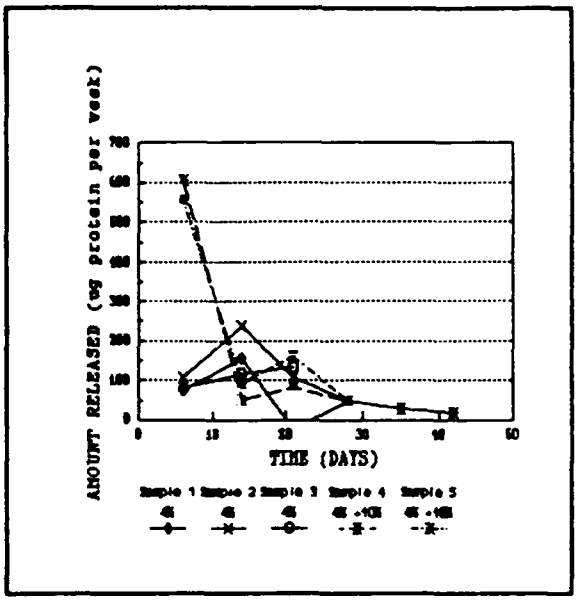


FIGURE 3.8 - RELEASE OF BSA FROM 4%\BSA\96%PLGA AND 4%BSA\10%AHH\86%PLGA



A weight of sample which would contain 2~4 mg protein was chosen for each set of leaching studies. Weighed aliquots of powders were placed in cellulose extraction thimbles. The thimbles were suspended in tubes containing 10 ml of phosphate buffered saline (PBS) pH 7.4 (Sigma Diagnostics 1000-3) with 0.001% sodium azide added. The tubes were maintained at 32° C with stirring. At predetermined intervals (weekly) either an aliquot of the leaching buffer was removed and replaced with fresh, or the entire buffer was replaced, and the removed buffer was analyzed for protein content by BioRad Microassay [18]. In Figure 3.7 it can be seen that protein was released from a 7.5 % loaded matrix powder in an initial burst. Following the burst, a brief dormant period at about 3 weeks was followed by a secondary burst of protein release at 6 weeks and a smaller burst at about 10 weeks. For this formulation, either it would be considered to contain both the initial vaccine dose and the sleeper dose, or the powder would require a wash step to remove surface material prior to use.

In the second experimental set, two formulations, each containing 4% BSA [one also containing 10% AHH] were compared. The results are shown in Figure 3.8. In the BSA/PLGA formulation, it can be seen that this formulation released some protein initially, building to a peak at two weeks, followed by rapid drop off in release. In

[18]Bradford, M.M., Analytical Biochemistry 12:248-254 (1976)

calculating total protein release from this formulation, not all material in the formulation could be accounted for in the leaching media. In the formulation containing 10%AHH, an initial burst was again observed, followed by a peaking of release at three weeks.
Our interpretation of these results are that: 1) The relationship of protein loading to burst time may be inverse; and 2) the addition of AHH to the formulation delays the time of the burst. The exact parameters for a specific vaccine will depend greatly on the exact nature of the antigen, however we anticipate that the ideal formulation will contain either no AHH or less than 10% AHH (prolonging the "sleeper" time),and less than 4% antigen.

3.3.3 Protein Stability

The concept was to prepare DNP-BSA/PLGA as previously described for BSA/PLGA at a 4% loading. The two systems would not be entirely similar, because of the differences in solubility of the modified protein preparation, but they were expected to be similar in that some protein would be released from the matrix immediately upon placement in buffer, and some would still be releasing from the matrix after two weeks in buffer.

To this end, 2.9994 g of PLGA was dissolved in 30 ml of methylene chloride and pressure filtered. We assumed 1% weight loss for the filtration resulting in 2.9694 grams polymer in the filtrate. An aliquot of 0.1233 g of DNP-BSA (Calbiochem Corp, 324101) was added along with glass beads. The suspension was ball milled overnight to reduce the particle size of the DNP-BSA. The suspension was cast under nitrogen, and dried, extruded, and ground as previously described, resulting in 0.8879 grams of product.

Two methods of protein analysis were employed: one was the BioRad Assay previously referenced; and the other was the ELISA method of competitive inhibition. In this method, we coated wells in the immunoassay plates with DNP-BSA; individual standards (DNP-BSA) and samples collected as described above, were incubated with antibody to DNP-BSA [anti-DNP-BSA-goat]. After incubation, the mixtures were added to the coated wells, and sufficient time was alloted for anti-DNP-BSA-goat which had not bound to DNP-BSA in the samples or standards to bind to that protein on the plate. After washing, so that only the bound anti-DNP-BSA-goat remains (the more DNP-BSA in the sample or standard the less binding on the plate occurs) the enzyme linked conjugate [alkaline phosphatase-anti-goat IgG (H&L)] was added. After incubation and washing the alkaline phosphatase substrate (Sigma No. 104-0) was added and the development of color was read on an ELISA reader. The results from the standards allowed calculation of the amount of DNP-BSA in the samples.

The methodology for this assay was developed as follows: (1) to find the proper dilution of antibody (anti-DNP-BSA-goat), we coated plates with a fixed amount (100 ul of a 50 ugm/ml solution) of DNP-BSA. We made serial dilutions of the antibody (anti-DNP-BSA-goat) and chose the one which, after substrate addition and color development,

gave ~50% of the absorbance range of the dilutions tested. The antibody dilution chosen (1:20,000) produced a reading of ~0.75 absorbance units; (2) to find the range of DNP-BSA concentrations for which the test was most sensitive, we used the antibody dilution determined in (1) and a range of DNP-BSA dilutions that showed linearity after incubation with anti-DNP-BSA-goat and further incubation and color development. The linear range was found to be 50 to 250 ng/ml.

We then designed the leaching sample preparation to obtain concentrations in the desired range. Planning was made to obtain samples for both the ELISA test and the BioRad Assay. Three samples of ~50 mgs were placed in 10.0 mls PBS and left at 37°C for 2 weeks. The buffer was removed, the particles rinsed with PBS, and 5.0 mls of fresh buffer were added. At the same time, three dry samples were placed in similar tubes and 10.0 ml of buffer was added to each of these. After 24 hours, the buffer was removed from each of the six samples. Thus one set of samples was used to collect protein from the matrix which had been in buffer for only 24 hr, while the other set provided protein which had been in wet matrix for 2 weeks. Each buffer sample was divided so that portions were used for each of the two assay types, and since the amount of protein collected was not known in advance, many sample dilutions were tested. The results, shown in Figure 3.10, illustrate that more protein released from the matrix in the first 24 hr period than in the 24 hrs +2 weeks; this is expected, given the results of the earlier BSA/PLGA experiment (Section 3.3.2).

Also the figures indicate that after processing the matrix and storage at room temperature, the ratio of ELISA ASSAY to BioRad assay is 67% $\pm$37.7% , indicating that a substantial percentage of DNP-BSA sites are retained. After 2 weeks, the ratio of ELISA results to Biorad results indicates that the proportion of DNP-BSA sites to initial is 24% $\pm$38%. The deviations on these samples are large, in part because of the difficulty of handling small samples, and because the protein levels were at the low end of the sensitivity of the Bio Rad test. The deviations were calculated as the square root of the sum of the squares of the individual assay standard deviations. When the 4 data sets (BioRad T=0 and T=2 weeks, and ELISA T=0 and T=2 weeks, are compared statistically by the Newman-Keuls test [19], the first and the last named data sets differ significantly with $p < 0.05$. The others are not significantly different.

In retrospect, it would have been valuable to prepare samples of free DNP-BSA which sat in buffer at 37°C for 24 hrs and 2 weeks plus 24 hrs. Because we do not know the rate of hydrolysis of the DNP-BSA bond under these conditions (pH 7.4, 37°C) we are able to say only that it appears that some losses do occur in the matrix. Some of the loss

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[19]Tallarida, R.M. and R.B. Murray, Manual of Pharmacologic Calculations, second edition, Springer-Verlag, 1986

may be due to loss of DNP as the solutions did become yellow. The test would need to be repeated with the actual vaccine of interest to determine percent loss.

FIGURE 3.9 - FLOW CHART FOR PROTEIN STABILITY STUDY

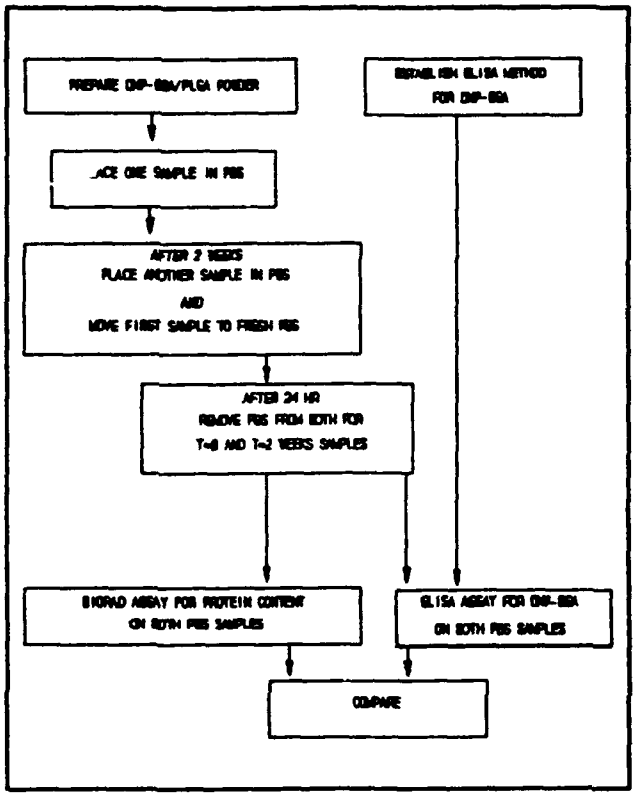
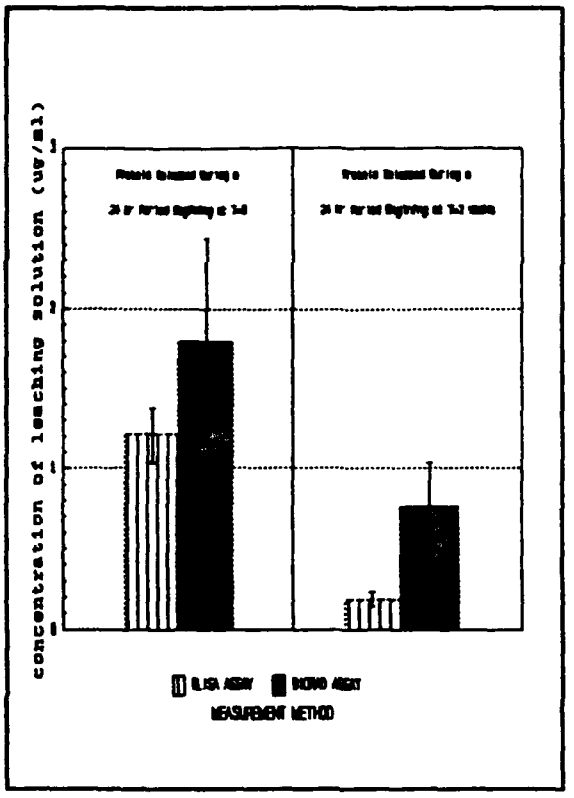


FIGURE 3.10 - RESULTS OF PROTEIN STABILITY STUDIES



Section 4

RECOMMENDATIONS FOR PHASE II

4.1 Overview

In Phase I, a non-antigen containing six week "sleeper" formulation and model protein containing 2-3 week "sleeper" formulations were identified. It is believed that continued work, focusing on the incorporation of the antigen actually targeted for product use is highly desirable. The prospects for success appear high, and the utility of the envisioned product, for the U.S. Army, for the U.S. civilian, and for others in less developed areas, is beyond doubt.

A two year program is envisioned for Phase II. The Phase II program objectives are: to prepare and demonstrate in vivo efficacy of a "sleeper" form of the HAV formulation in the stimulation of antibody production; to carry out toxicological testing; and to submit to the U.S.Army documentation to support an amendment to the present HAV vaccine IND for clinical evaluation of the "sleeper" formulation.

4.2 Outline of Phase II Workplan

The pathway of the Phase II program will lead the investigators past the following milestones:

1. Demonstration that processing does not inactivate antigen;
2. Preparation of batches of 3 initial formulations plus one blank [at least 100 doses of each];
3. Validation of the 3 initial formulations;
4. Preparation of sets of refined formulations;
5. Completion of in vivo tests to measure anti HAV titers in the refined formulations;
6. Completion of validation and Toxicity Testing on the final formulation;
7. Compilation of documentation for IND addendum.

The complete proposed program description is given in a separate document.